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Inhibitory Effect of Crude Ethanol and Water Extracts of *Phytolacca dodecandra* (L' Herit) on Embryonic Development of *Anopheles gambiae* (Diptera: Culicidae)

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Abstract

Plant extracts have been demonstrated to interfere with activities of juvenile hormones that facilitate egg hatching by the embryo. In the present study we demonstrate ovicidal effect of crude ethanol and water extracts of mature green fruits and leaves of *Phytolacca dodecandra* against *Anopheles gambiae* eggs under ambient laboratory condition. Freshly laid *An. gambiae* eggs were exposed to 33.33mls of water and ethanol extracts of mature green fruits and leaves of shoot and midsections of *P. dodecandra*. The solutions were a serial dilution of 40, 20, 10, 5 and 5mg/100mls of the crude extracts dispensed in plastic containers measuring 6cm top × 5.7cm bottom × 3.5cm height. Ovicidal activity was assessed 48 hours post exposure under dissecting microscope (Leica Zoom 2000) at ×10 magnification. Unhatched eggs were judged dead when non-hatched and with unopened opercula and alive when hatched or with open operculum. WHO threshold of > 80 % mortality was used to assess ovicidal effectiveness. Crude water extracts of leaves of the shoots killed more than 80% while ethanol extracts of the same parts killed 60% of exposed eggs. Leaf extracts of the shoots were more effective compared to extracts of leaves of midsection and mature green fruits. Egg mortality was higher for *P. dodecandra* extracts compared to Neem or deltamethrin. It was concluded that water extracts were more potent as ovicides than ethanol extracts irrespective of part of *P. dodecandra* used. Extracts of *P. dodecandra* can be used as an alternative to chemical insecticide in malaria vector control.

Keywords: Anopheles gambiae, Deltamethrin, Ethanol, Neem, *Phytolacca dodecandra*

1. Introduction

Mosquitoes are vectors of medically important viruses (Polwiang, 2015; Ghimire and Dhakal, 2015: Kindhauser *et al.*, 2016) and malaria (Beck-Johnson *et al.*, 2013; Kumar *et al.*, 2013) and as such are a threat to public health. However, the war against mosquitoes using chemical insecticides has failed due to development of resistance strains (Aizoun *et al.*, 2013; Jones *et al.*, 2013; Stenhouse *et al.*, 2013; Nardini *et al.*, 2013; Vannini *et al.*, 2014) . In addition, the pesticides have been found to negatively impact human health and environment as they are non-biodegradable and some even bioaccumulate (Cartilla and De la Cruz, 2012). For these reasons, research for new control tools is focused on biopesticides and botanical formulations, which are considered eco-friendly, pest specific, biodegradable (Bowers, 1992), and composed of novel compounds with a wide range of activities (Ghosh *et al.*, 2008). The plant-borne compounds are also effective in small doses (Benelli, 2015; Pavela, 2015) and can be employed for rapid synthesis in nanoformulations (Benelli, 2016).

In recent years, plant products and phytochemicals have been evaluated against different developmental stages of mosquitoes, either on individual mosquito stage or a combination (Shaalam *et al.*, 2005; Ivoke, 2005; Chansang *et al.*, 2005; Mehlhorn *et al.*, 2005; Amer and Mehlhorn, 2006; Gleiser and Zygadlo, 2007; Govindarajan *et al.*, 2008; Muthu *et al.*, 2012; Kaliyamoorthy *et al.*, 2012; Ramar *et al.*, 2014; Valentina *et al.*, 2015). Plant extracts have also been found efficacious as growth inhibitors against eggs of different mosquito strains. For instance; *Boswellia dalzielii* (Younoussa *et al.*, 2016) and *Ocimum basilicum* (Foko *et al.*, 2016) against *An. gambiae*, *Spathodea campanulata* (Pravin *et al.*, 2015), *Argemone Mexicana* (Warikoo and Kumar, 2015) against *Ae. aegypti*, *Boswellia dalzielii* (Younoussa *et al.*, 2016) against *Culex quinquefasciatus* (Say) and *Melanochyla fasciculiflora* leaf and *Gluta renghas* leaf (Zuharah *et al.*, 2015) against *Aedes albopictus*.

Phytolacca dodecandra a biopesticide, has demonstrated potency against snail vectors of bilharzias (Erko *et al.*, 2002; Abebe *et al.*, 2005), filarial vector *Culex quinquefasciatus* Say (Misganaw *et al.*, 2012), *An. gambiae* larvae (Yugi *et al.*, 2015) as well as adults (Yugi *et al.*, 2014; 2016) with impressive outcome. Nonetheless, despite these positive results, there is a dearth of literature report on its inhibitory effect on *An. gambiae* embryo

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development. It was with respect to this that the present study was designed to evaluate and report on the activity of ethanol and water extracts of *P. dodecandra* on *An. gambiae* eggs under laboratory condition.

2. Materials and Methods

2.1. Study Area, Experimental Mosquitoes and Study Design

The experiments were conducted in the Entomology laboratory at the Centre for Global Health Research / Kenya Medical Research Institute (CGHR/KEMRI). *An. gambiae* mosquitoes, maintained in the laboratories and reared following standard procedures (Das *et al.*, 2007), were used in the experiments. A randomized informal 'after-only with control' experimental design (Kothari, 2004) was used to investigate the embryonic inhibitory effect of crude ethanol and water extracts of *P. dodecandra* on the eggs. The climatic conditions within the insectary and the laboratory were 28 – 30°C temperatures 70 - 80% relative humidity and 12:12, L:D photoperiod (Yugi *et al.*, 2014).

2.2. Deltamethrin (KOTab 1-2-3®) and Plant Materials Acquisition and Preparation

Deltamethrin (KOTab 1-2-3®) was acquired and prepared as described (Yugi *et al.*, 2015). Fresh leaves (shoot and midsection) and mature green fruits of *P. dodecandra* and fresh leaves of *Azadirachta indica* (Neem), were acquired, identified and voucher specimen deposited as earlier described (Yugi *et al.*, 2015). The plant parts were shade dried at room temperature, grounded and extracts obtained using ethanol and water following Tilahun *et al.* (2003); Parekh *et al.* (2005) and Das *et al.* (2010) procedures.

2.3. Ovicidal Assays

A modified method of Elango *et al.* (2009) was used to determine the embryonic inhibitory activity of crude ethanol and water extracts of green mature fruits and leaves (shoot and midsection) of *P. dodecandra* on *An. gambiae* eggs. Freshly laid eggs were collected from the entomology laboratory at CGHR/KEMRI, counted in batches of 20 eggs each from filter papers under a dissecting microscope (Leica Zoom 2000) at ×10 magnification using fine tipped painting brushes and placed in smaller Whitman No. 1 filter papers. Each of such filter paper was then placed in plastic containers (Figure 1) containing approximately 33ml of a given treatment.

Figure 1. Plastic containers used in exposing eggs of *An. gambiae*

The treatments were prepared by weighing 80 milligrams of stock's crude extracts of *P. dodecandra* and dissolving this in 100 millilitres of rain water. The resulting solution was then serially diluted to different concentrations of 40, 20, 10, 5 and 2.5 mg/100ml. A solution of a particular concentration was later distributed among a set of three plastic containers. Each plastic container held an approximate volume of 33mls. The sets of containers were arranged in ascending order with respect to concentration of solutions therein. The arrangement was repeated for similar concentrations of Neem and deltamethrin. Extracts of Neem and solutions of deltamethrin were used as positive control while exposure to rain water alone as negative control. The experimental set ups were replicated five times for each treatment with appropriate controls.

Embryonic inhibitory activity was assessed 48 hours post treatment by observing the eggs under dissecting microscope (Leica Zoom 2000) at ×10 magnification. Egg hatchability was scored as a function of hatched larvae or unhatched but live egg. Unhatched egg was judged as live and capable of hatching or dead and incapable of hatching from observation. A dead eggs appeared non-hatched and with unopened opercula while live egg was one that appeared unhatched but with open operculum. Dead eggs were counted for each treatment, and the percent mortality calculated using a modified version of Abdullahi *et al.* (2011) formula:

$$\%\ \text{egg mortality} = \frac{\text{Total number of eggs exposed} - \text{Number of hatched larvae}}{\text{Total number of eggs exposed}} \times 100$$

Bioassay tests showing more than 20% control mortality were discarded and repeated. However, when control mortality ranged from 5% to 20%, the corrected mortality was calculated using Abbott's formula (Abbott, 1925):

$$\text{Corrected mortality} = \frac{\%\ \text{test mortality} - \%\ \text{control mortality}}{100 - \%\ \text{control mortality}} \times 100$$

Standard WHO procedure was used to assess effectiveness of the extracts as an ovicide at a mortality rate of > 80% (WHO, 2005).

2.4. Data Analysis

Data obtained from the bioassays were analysed using one way analysis of variance (ANOVA). Regression (probit) analysis was used to calculate the lethal concentration (LC_{50}) and χ^2 statistics of the extracts used. The means were separated using LSD and differences between them as well as that of χ^2 statistics considered significant at $P < 0.05$. All statistical analysis was performed using SAS statistical package version 20. .

3. Results

This experiment was conducted for a period of two months using 10, 800 freshly laid *An. gambiae* eggs. It was found that crude water and ethanol extracts of leaves and shoot of *P. dodecandra* inhibited >80% and <60% hatching of the exposed eggs respectively. Extracts of *P. dodecandra* leaves from shoots recorded a higher percentage of unhatched eggs than extracts of mature

green fruits or leaves of the midsection. Solvent used in extraction did not influence embryonic inhibitory effect of *P. dodecandra* extracts as numbers of unhatched eggs from exposures were close. Extracts of *P. dodecandra* were more potent as embryonic inhibitors compared to statistics on extracts of Neem leaves or deltamethrin (Figure 2).

Activity of the extracts was dose dependent with activity reducing with reduced concentration irrespective of source or solvent used. Water extracts of *P. dodecandra* were more potent than ethanol extracts regardless of concentration or source of *P. dodecandra* plant (Figure 3). Extracts of *P. dodecandra* sourced from the Highlands (Eldoret) were more potent than those sourced from the lowlands (Nyando), though the influence of extracts of *P. dodecandra* from the two sources on embryonic inhibitory activity was similar (Table 1). Overall, extracts of *P. dodecandra* were more potent as embryonic inhibitors compared to extracts from Neem and deltamethrin (Table 2). Calculated estimates of the lethal concentration (LC_{50}) of *P. dodecandra* extracts were relatively low irrespective of parts or solvent used. Estimated values for water extracts were lower than those of ethanol extracts and even that of deltamethrin. All calculated χ^2 values were less than the χ^2 critical value of 22.362 (df = 13; $p < 0.05$). This means that the H_0 hypothesis of no relationship between dose and mortality of *An. gambiae* eggs was retained. The influence of the lethal concentrations (LC_{50}) of *P. dodecandra* on the inability of the exposed *An. gambiae* egg to hatch did not however, significantly differ (Table 3).

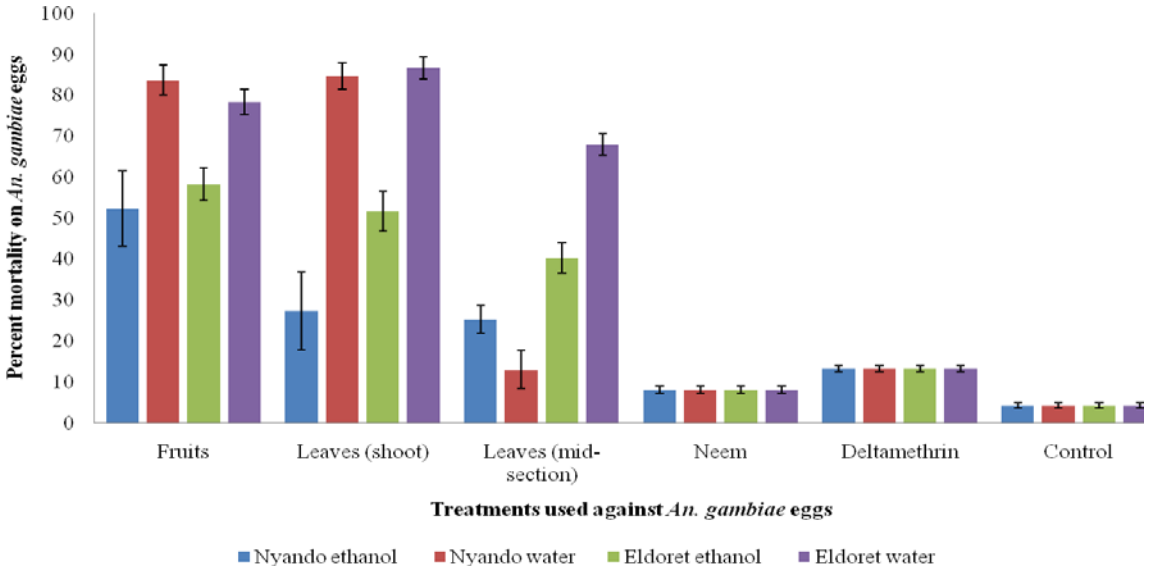

Figure 2. Ovicidal effects of ethanol and water extracts of different parts of *P. dodecandra* sourced from Eldoret and Nyando against *An. gambiae* eggs

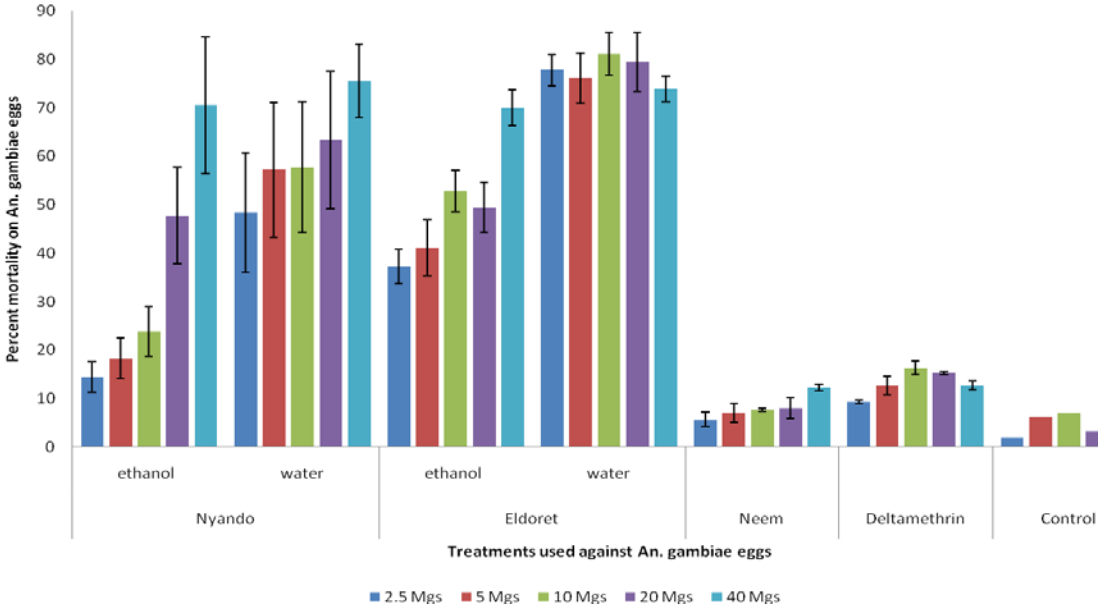

Figure 3. Ovicidal efficacies of different concentrations of ethanol and water extracts of *P. dodecandra* from Eldoret and Nyando against *An. gambiae* eggs

Table 1. Comparative of regional effectiveness of ethanol and water extracts of *P. dodecandra* against *An. gambiae* eggs.

***P. dodecandra* source**	**df**	**Solvent used in extraction**			
		Water		**Ethanol**	
		F	P	F	*P*
Nyando	4	167.687	0.000	7.919	0.000
Eldoret	4	285.249	0.000	47.838	0.000

Notes: df stands for degree of freedom; F stands for the F statistical factor; P stands for probability for the level of significance. *P* was taken as significant at $p < 0.05$

Table 2: Duncan's Statistics on ovicidal effectiveness of different treatments against *An. gambiae* eggs.

Treatments	df	F	*P*	F	*P*
P. dodecandra (Nyando)	4	0.636	0.640	8.044	0.000
P. dodecandra (Eldoret)	4	0.397	0.810	7.729	0.000
Neem	4	2.869	0.080	2.869	0.080
Deltamethrin	4	6.000	0.010	6.000	0.010
Control	4	5.859	0.011	5.859	0.011

Notes: 1. df stands for degree of freedom; F stands for the F statistical factor; *P* stands for probability for the level of significance. P was taken as significant at $p < 0.05$

Table 3: Estimated lethal concentration (LC_{50}) of ethanol and water extracts of *P. dodecandra* used against *An. gambiae* eggs. The estimated are reported together with standard errors (SE).

Phytolacca dodecandra		df	Solvent of extraction					
			Ethanol			Water		
Source	Parts used		LC_{50}± SE	χ^2	p	LC_{50}± SE	χ^2	p
Eldoret	Fruit	13	8.11± 0.18	5.879	0.950	1.45± 4.19	18.005	0.157
	Leaves of mid-section	13	10.54± 0.18	4.588	0.983	2.30± 0.18	3.152	0.997
	Leaves of shoot	13	13.50± 0.18	4.921	0.977	1.70± 0.28	10.221	0.676
Nyando	Fruit	13	8.97± 0.25	15.976	0.250	1.74± 0.29	6.072	0.943
	Leaves of mid-section	13	1.74± 0.29	6.072	0.943	24.09± 0.20	3.191	0.997
	Leaves of shoot	13	11.26± 0.24	12.89	0.546	1.62± 0.25	10.029	0.692
Control	Neem	13	19.31± 0.19	4.505	0.985	5.39± 0.21	9.466	0.737
	Deltamethrin	13	2.52± 0.19	7.562	0.871	2.52± 0.19	7.562	0.871

Notes: 1. df = degrees of freedom (n-2); 2. χ^2 = chi-square test statistics of relationship between the considered factors; *p* = level of significance. The relationships were considered significant at $p<0.05$; SE = standard error

4. Discussion

The present study demonstrates the following on effectiveness of ethanol and water extracts of *P. dodecandra* plant parts as ovicides. First, it has an inhibitory effect on *An. gambiae* mosquito embryo growth and development. Second, the effectiveness of the extract is dose dependent and that potency reduces with reduced concentration irrespective of plant part or solvent used. Third, water is the best solvent for extracting bioactives of *P. dodecandra* as water extract were more potent than ethanol extract irrespective of plant part used. Lastly, extract of *P. dodecandra* leaves are more potent than that of mature green fruits.

Plant extracts such as *Pemphis acidula* (Samidurai *et al*., 2009)**,** *C. celata* (Reegan *et al.*, 2013) on *Cu. quinquefasciatus* and *An. aegypti* eggs, *Exacum pedunculatum* on *An. stephensi* (Elangovan *et al*., 2012) to mention but a few have been evaluated against mosquito eggs and found potent. However, no report on embryonic inhibitory activities for any part of *P. dodecandra* is available against any mosquito species. The present study reports and demonstrates that concentrations of 20 mgs/100mls and higher of ethanol and water extracts of mature green fruits and leaves of *P. dodecandra* are potent against *An. gambiae* eggs as they hindered more than 80% of the exposed *An. gambiae* eggs irrespective of solvent used or geographical source of *P. dodecandra* plants from hatching. The effectiveness however, reduced with reduced concentration. This showed that effectivity of *P. dodecandra* extracts as ovicides were dose dependent an observations that correlated with those of Elumalai *et al.* (2010); Ramar *et al.* (2014); Younoussa *et al.* (2016) and Foko *et al.* (2016) who reported the efficacy of various essential oils and leaf extracts against *Culex quinquefasciatus* eggs, *Spodoptera litura* and *Anopheles gambiae*, respectively

These findings place *P. dodecandra* extracts among such products as essential oils from three spontaneous plants against *Aedes aegypti* and *Anopheles gambiae* complex (Bassolé *et al.*, 2003), *Hyptis suaveolens* leaf extracts against *Anopheles gambiae* (Ivoke *et al.*, 2009) and *Cardiospermum halicacabum* leaf extract against *Culex quinquefasciatus* and *Aedes aegypti* (Govindarajan, 2011) among others. It also places *P. dodecandra* extracts in the same rating as methoxyfenozide that has been demonstrated not only to disrupt growth but also to delay development in *Culex pipiens* L. (Hamaidia and Soltani, 2016).

In the present study, solvent played a significant role in shaping activity of the extracts. Water extracts were more potent than ethanol extracts. *P. dodecandra* bioactives also seemed to distribute differentially along the vertical stretch of the plant with the leaves having more potent bioactives than the green fruits. These findings were consistent with earlier observation that reported a correlation of levels of potency of plant extracts with type of plant part and solvent used in the extraction of bioactives (Jeyabalan *et al.*, 2003; Mgbemena, 2010; Anupam *et al.*, 2012).

P. dodecandra extracts from the highlands (Eldoret) were more potent than those from the lowlands (Nyando) irrespective of the parts used. This observation demonstrates that spatial disposition of a plant influences the concentration of bioactives within and that plants from different geographical zones have bioactives of different potency. The differential potency of bioactives of *P. dodecandra* from different ecological regions reported in this study is consistent with that of Anupam *et al.* (2012)

and Mgbemena, (2010) that demonstrated that geographical source influences level of potency of bioactives in plant samples.

The fact that higher concentrations of *P. dodecandra* extracts killed >80% of exposed *An. gambiae* eggs, demonstrates that the extracts are effective as ovicides (WHO, 2005). These observations demonstrate without doubt that *P. dodecandra* extracts are potent inhibitors of mosquito embryo development and are in the same rank as insect growth disruptor as methoxyfenozide (Hamaidia and Soltani, 2016). The mechanism through which the embryos died may not be known as this was not investigated by the present study but it is speculated it could have been due to blockage of the micropyle thereby interfering with gaseous exchange or by interfering with purity of the gases within the operculum preventing the embryo from accessing air and thus ultimately dying (Bhatnagar and Sharma, 1994).

Eggs play a crucial role in the life cycle of insects as they are the starting point of new generation and their correct development and timely hatching is important. Studies have shown that egg hatchability in insects and most importantly *An. gambiae* is influenced by several factors among which are temperature, salinity and humidity (Lyons *et al.*, 2013), organic substances and bacteria (Lindh *et al.*, 2008; Sumba *et al.*, 2008). Delay or reduction in the rate of growth and development or even death to the eggs definitely affects individual fitness, population structure and dynamics (Spencer *et al.*, 2002). It is therefore understandably that anything that interferes with the correct development of this stage (eggs) of malaria vector greatly impact malaria transmission and morbidity.

In conclusion, the present study report demonstrates two things. First, that water extracts of *P. dodecandra* are more potent as ovicides than ethanol extract irrespective of part used and second, that extracts of *P. dodecandra* interferes with embryo development in *An. gambiae* leading to inability of the eggs to hatch. It is recommended that extracts of *P. dodecandra* be used as an alternative to chemical insecticide in malaria vector (*An. gambiae* mosquito) control.

Authors' Contributions

YJO conceived the concept, designed, sourced for funds and wild mosquito larvae and wrote the manuscript. YJO and KJJ, conducted the experiments, read and corrected the manuscript.

Competing Interests

The authors declare that they have no competing interests and that Neem and Deltamethrin were used purely for experimental purpose.

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